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# Molecular Evidence for Hierarchical Transcriptional Lineage Priming in Fetal and Adult Stem Cells and Multipotent Progenitors

Robert Månsson,[1,3] Anne Hultquist,[1,3] Sidinh Luc,[1,3] Liping Yang,[1,3] Kristina Anderson,[1] Shabnam Kharazi,[1] Suleiman Al-Hashmi,[1] Karina Liuba,[1] Lina Thorén,[1] Jörgen Adolfsson,[1] Natalija Buza-Vidas,[1] Hong Qian,[1] Shamit Soneji,[2] Tariq Enver,[2] Mikael Sigvardsson,[1] and Sten Eirik W. Jacobsen[1,*]

[1] Hematopoietic Stem Cell Laboratory, Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, Lund University, 221 84 Lund, Sweden

[2] MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford, OX3 9DS, United Kingdom

[3] These authors contributed equally to this work.

*Correspondence: sten.jacobsen@med.lu.se

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## SUMMARY

**Recent studies implicated the existence of adult lymphoid-primed multipotent progenitors (LMPPs) with little or no megakaryocyte-erythroid potential, questioning common myeloid and lymphoid progenitors as obligate intermediates in hematopoietic stem cell (HSC) lineage commitment. However, the existence of LMPPs remains contentious. Herein, global and single-cell analyses revealed a hierarchical organization of transcriptional lineage programs, with downregulation of megakaryocyte-erythroid genes from HSCs to LMPPs, sustained granulocyte-monocyte priming, and upregulation of common lymphoid (but not B and T cell-specific) genes. These biological and molecular relationships, implicating almost mutual exclusion of megakaryocyte-erythroid and lymphoid pathways, are established already in fetal hematopoiesis, as evidenced by existence of LMPPs in fetal liver. The identification of LMPPs and hierarchically ordered transcriptional activation and downregulation of distinct lineage programs is compatible with a model for HSC lineage commitment in which the probability for undergoing different lineage commitment fates changes gradually when progressing from HSCs to LMPPs.**

## INTRODUCTION

The integrity of the hematopoietic system depends on a large number of blood cell lineages being continuously replenished from a rare population of pluripotent hematopoietic stem cells (HSCs), representing a paradigm for how multilineage diversity can be achieved from a common stem cell through lineage commitment and subsequent differentiation (Kondo et al., 2003).

Although extensive knowledge has been obtained with regard to the identity and mechanisms of actions of regulators of blood lineage differentiation (Laiosa et al., 2006), the regulation of HSC lineage commitment remains elusive. A key step toward identifying such regulators is to identify the cellular pathways or intermediates in the HSC lineage commitment or lineage restriction process. The prevailing model for mammalian hematopoiesis predicts that commitment to a single hematopoietic lineage necessitates that HSCs first pass through a number of obligatory restriction sites or intermediate multipotent progenitors (Reya et al., 2001). Such a model has been strongly supported by the identification and characterization of distinct common lymphoid and common myeloid progenitors (CLPs and CMPs, respectively) (Akashi et al., 2000; Kondo et al., 1997), leading to the proposal that the first lineage commitment step of pluripotent HSCs results in a strict separation into common lymphoid and common myeloid pathways (Reya et al., 2001). However, this model hinges on all upstream multipotent stem and progenitor cells possessing the full repertoire of lineage potentials. Although this has been unequivocally established to be the case for long-term repopulating HSCs (LT-HSCs) (Osawa et al., 1996) and has been assumed to pertain also to short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs) (Reya et al., 2001), more recent findings have strongly implicated the existence of MPPs with combined granulocyte-macrophage (GM) and lymphoid potentials, but little or no megakaryocyte-erythroid potential (MkE) (Adolfsson et al., 2005; Lai et al., 2006; Yoshida et al., 2006), within the adult mouse Lineage$^-$SCA-1$^+$KIT$^+$ (LSK) HSC compartment. In contrast, there are as of today no reports supporting the existence of stem or progenitor cells with combined lymphoid and MkE but no GM potential.

The proposed existence of LSKCD34$^+$FLT3$^{hi}$ lymphoid-primed MPPs (LMPPs), questioning the classical CMP-CLP model as an obligatory route for lineage commitment (Adolfsson et al., 2005), has been cast into doubt by

others (Forsberg et al., 2006), because a fraction of LSKCD34$^+$FLT3$^{hi}$ bone marrow (BM) cells have some residual MkE potential. Specifically, single-cell in vitro studies revealed that very rare (2%–3%) LSKCD34$^+$FLT3$^{hi}$ cells had MkE potential (Adolfsson et al., 2005), whereas others demonstrated, when transplanting high cell numbers (>500 cells per mice), that BM LSKCD34$^+$FLT3$^{hi}$ cells had some in vivo MkE potential (Forsberg et al., 2006). The LSKCD34$^+$FLT3$^{hi}$ population, like any stem and progenitor cell population, is heterogeneous, so it therefore remains unclear whether this reflects that an infrequent progenitor cell within the LSKCD34$^+$FLT3$^{hi}$ population sustains MkE potential, unlike LMPPs, or whether LMPPs have some residual MkE potential. Thus, there is a need to further purify, or identify and characterize LMPPs based on alternative and more direct investigations, rather than exclusively based on their lineage output in biological assays.

At variance with committed progenitors, HSCs and MPPs express (at the single-cell level) multiple lineage-associated genetic programs, proposed to reflect their intrinsic multilineage potentiality (Akashi et al., 2003; Hu et al., 1997; Miyamoto et al., 2002). Importantly, if the succession from LT-HSCs through ST-HSCs and MPPs, prior to generation of the CLPs and CMPs, would be accompanied exclusively by changes in self-renewing ability and not in lineage potentials (Reya et al., 2001), the multilineage genetic priming of LT-HSCs, ST-HSCs, and MPPs would be expected to remain similar. Thus, herein we explored the transcriptional lineage priming of recently identified, phenotypically and functionally distinct and hierarchically related LSK subsets in adult BM: LSKCD34$^-$FLT3$^-$ LT-HSCs, LSKCD34$^+$FLT3$^-$ ST-HSCs, and LSKCD34$^+$FLT3$^{hi}$ LMPPs (Adolfsson et al., 2005; Yang et al., 2005). Global gene profiling of these populations revealed compelling molecular evidence for a distinct hierarchical organization of lineage programs, with a gradual downregulation of MkE transcriptional priming from LT-HSCs to LMPPs, sustained GM priming, and a distinct upregulation of common lymphoid genes in the LMPPs. Analysis of early-stage hematopoietic development in the fetal liver (FL) revealed a similar hierarchy and that the LMPPs are defined already at this early stage of development.

## RESULTS

### LMPPs Are Defined during Fetal Development

Although the existence of lineage restricted lymphoid-GM progenitors has also been implicated in fetal development (Cumano et al., 1992; Lacaud et al., 1998; Lu et al., 2002), it has been suggested that the earliest lineage restriction decisions of HSCs might differ in FL and adult BM (Katsura, 2002). To investigate whether the HSC hierarchy contains a phenotypically and functionally distinct LMPP population already during fetal development, we analyzed the LSK compartment in 14.5 days postcoitum (dpc) FL. Although LT-HSCs in FL, in contrast to BM, have been demonstrated to be CD34$^+$ (Ogawa, 2002), we noticed that a small fraction of LSK FL cells expressing low CD34 were predominantly FLT3$^-$ (Figure 1A), as observed for LSKCD34$^-$ cells in BM (Figure S1 in the Supplemental Data available online). Thus, FL LSK cells were purified by FACS into LSKCD34$^{lo}$FLT3$^-$, LSKCD34$^{hi}$FLT3$^-$, and LSKCD34$^+$FLT3$^{hi}$ cells (Figure 1A). In addition, we also sorted a fourth population of LSKCD34$^+$FLT3$^{lo}$ cells to investigate its relationship to the LSKCD34$^+$FLT3$^{hi}$ population.

We first performed in vivo reconstitution studies, primarily to determine which of these four populations contained HSC activity. This was particularly important, because previous studies had suggested that LT-HSCs in FL were in part LSKFLT3$^+$ (Christensen and Weissman, 2001). Transplantation of as few as 40 cells of each population in competition with 200,000 unfractionated BM cells established that the FL LSKCD34$^{lo}$FLT3$^-$ population was highly enriched in cells with long-term multilineage reconstituting activity in all primary (Figure 1B), as well as secondary recipients (Figure 1C). As for mice transplanted with FL LSKCD34$^{hi}$FLT3$^-$ cells, some but not all mice sustained long-term multilineage reconstitution in primary recipients (Figure 1B), but only in one of these was secondary reconstitution observed (Figure 1C). The remaining recipients reconstituted well in the short and mid-term, but myeloid reconstitution was undetectable after 16 weeks and in secondary recipients. In other experiments (Figure S2), the separation in long-term multilineage reconstituting activity between LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$ cells was even more distinct, suggesting that LT-HSCs are most highly enriched in FL LSKCD34$^{lo}$FLT3$^-$ cells. Although the FL LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$ populations reconstituted the GM lineage in the short-term, they did this with less efficiency and durability than LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$ cells and showed no long-term multilineage repopulating ability (Figure 1B). However, they were relatively more efficient at rapidly reconstituting B and particular T lymphopoiesis than FL LSKCD34$^{hi}$FLT3$^-$ cells (Figure 1B), as previously demonstrated for adult LSKCD34$^{hi}$FLT3$^{hi}$ cells (Yang et al., 2005), identifying them as candidate fetal LMPPs without LT-HSC activity.

Although in vivo reconstitution assays are important in establishing whether LSK subsets have LT-HSC potential and ability to in vivo reconstitute different blood cell lineages, they are not optimized to establish, at the single-cell level, the potential of short-term multipotent progenitors to produce each of the myeloid and lymphoid lineages. Thus, we next explored the candidate FL LMPPs, and in comparison the three other FL LSK populations, for their extent of detectable Mk, E, G, M, B, and T cell potentials in clonal single-cell in vitro assays. Initially, because the kinetics and conditions optimal for generation of different lineages vary, each of these lineage potentials was first investigated individually. In agreement with representing populations containing LT-HSCs and ST-HSCs, single FL LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$ cells generated each of the myeloid (Mk, E, G, and M) lineages with very high efficiencies (Figures 2A and 2B), whereas the B and T cell potentials of LSKCD34$^{lo}$FLT3$^-$ (39% and 37%, respectively) and LSKCD34$^{hi}$FLT3$^-$

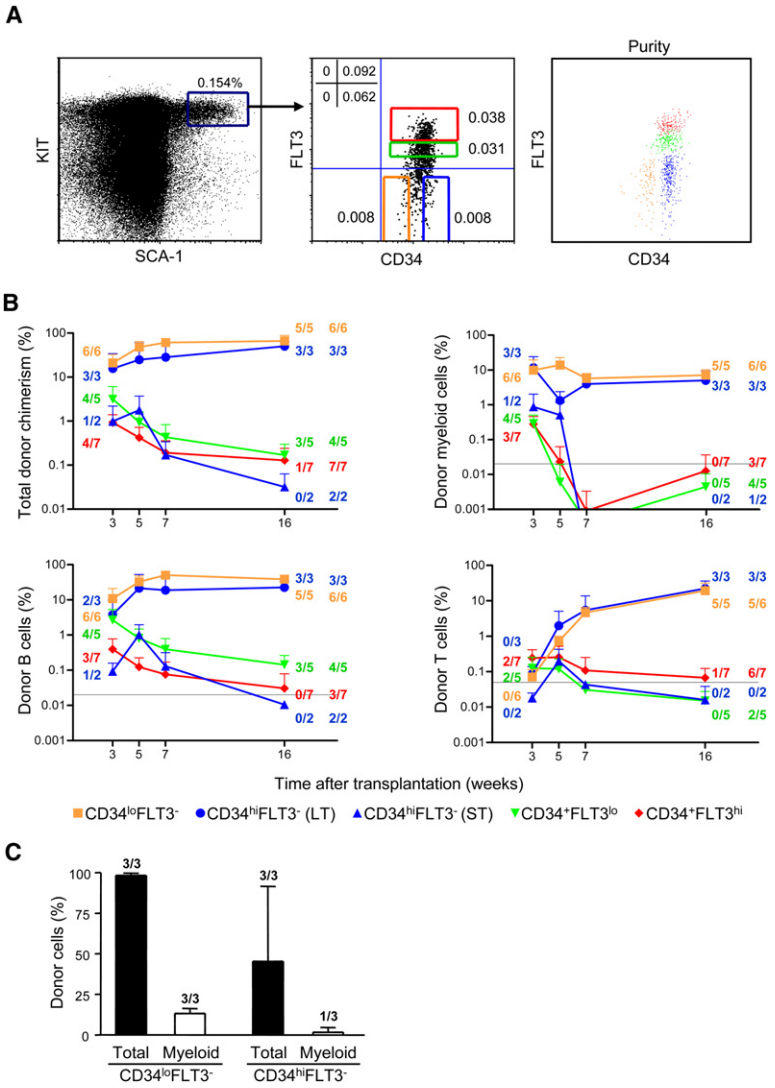


**Figure 1. Distinct Multilineage Reconstitution Patterns of FL LSK Subsets**

(A) Boxes denote the sorting strategy used for each of the LSKCD34$^{lo}$FLT3$^{-}$, LSKCD34$^{hi}$FLT3$^{-}$, LSKCD34$^{+}$FLT3$^{lo}$, and LSKCD34$^{+}$FLT3$^{hi}$ populations (numbers indicate percentages of total FL cells), resulting in 96% or higher purity (right, composite figure of reanalysis of 4 populations).

(B) Lethally irradiated C57Bl/6 mice were transplanted with 40 cells of each LSK subpopulation in competition with 200,000 unfractionated BM cells. Panels show mean ± SD percent contribution toward total, myeloid, B cell, and T cell reconstitution in peripheral blood derived from transplanted LSKCD34$^{lo}$FLT3$^{-}$, LSKCD34$^{hi}$FLT3$^{-}$ (divided into mice revealing LT-HSC activity and no LT-HSC activity), LSKCD34$^{+}$FLT3$^{lo}$, and LSKCD34$^{+}$FLT3$^{hi}$ cells at 3, 5, 7, and 16 weeks after transplantation. Numbers indicate the frequencies of reconstituted mice at 3 and 16 weeks after transplantation (next to graphs), as well as frequencies of mice reconstituted at any time point (far right).

(C) Total and myeloid reconstitution of secondary recipients 12 weeks after transplantation of half a femur equivalent of BM cells from primary recipients with long-term multilineage reconstitution. Graphs show the mean ± SD percent, and numbers indicate frequency of reconstituted mice.

(28% and 47%, respectively) cells were somewhat lower than what could be expected. However, virtually all FL LSKCD34$^{lo}$FLT3$^{-}$ and LSKCD34$^{hi}$FLT3$^{-}$ cells generating visible clones on OP9 and OP9-DL1 stroma (see Experimental Procedures) did in fact generate B and T cells, respectively (Figure 2). Thus, the limited clonal growth in these assays could reflect the challenge of developing efficient stromal assays supporting the growth of primitive stem and progenitor cells, as previously suggested by others (Katsura, 2002). FL LSKCD34$^{+}$FLT3$^{lo}$ and LSKCD34$^{+}$FLT3$^{hi}$ cells generated not only G and M but also B and T cells at very high frequencies (Figures 2C and 2D), whereas only a small fraction of LSKCD34$^{+}$FLT3$^{lo}$ and virtually no LSKCD34$^{+}$FLT3$^{hi}$ cells generated Mk and E progeny, as previously demonstrated for adult LMPPs (Adolfsson et al., 2005). Importantly, the inability of most FL LSKCD34$^{+}$FLT3$^{hi}$ cells to generate Mk and E cells was verified through careful kinetic studies (Figure S3), and in the case of E potential through the use and comparison of two different methods for detection of E potential (May-Grünwald Giemsa [MGG] and 2,7-diaminofluorene [DAF] staining) (Figure 2 and Figure S4). Thus, as their adult counterparts (Adolfsson et al., 2005), fetal LSKCD34$^{+}$FLT3$^{hi}$ cells have extensive B cell, T cell, and GM but almost no Mk and E potential.

Although all the B, T, and GM potentials of BM LSKCD34$^{+}$FLT3$^{hi}$ LMPPs were not evaluated from the same single cells, previous studies could establish the existence of LMPPs within the adult LSKCD34$^{+}$FLT3$^{hi}$ compartment, although not their exact frequency (Adolfsson et al., 2005). Thus, to better evaluate the full combined B cell, T cell, and GM potentials of fetal LSKCD34$^{+}$FLT3$^{hi}$ cells, we developed a new assay in which we could simultaneously evaluate these potentials of single sorted FL LSKCD34$^{+}$FLT3$^{hi}$ cells with high efficiency (Figure 3A; Experimental Procedures). After initial cloning on the OP9 stromal cell line to promote B cell development (Vieira and Cumano, 2004) and subsequent culture on OP9-DL1 cultures to reveal T cell potential (Schmitt and Zuniga-Pflucker, 2002), as many as 79% of single FL

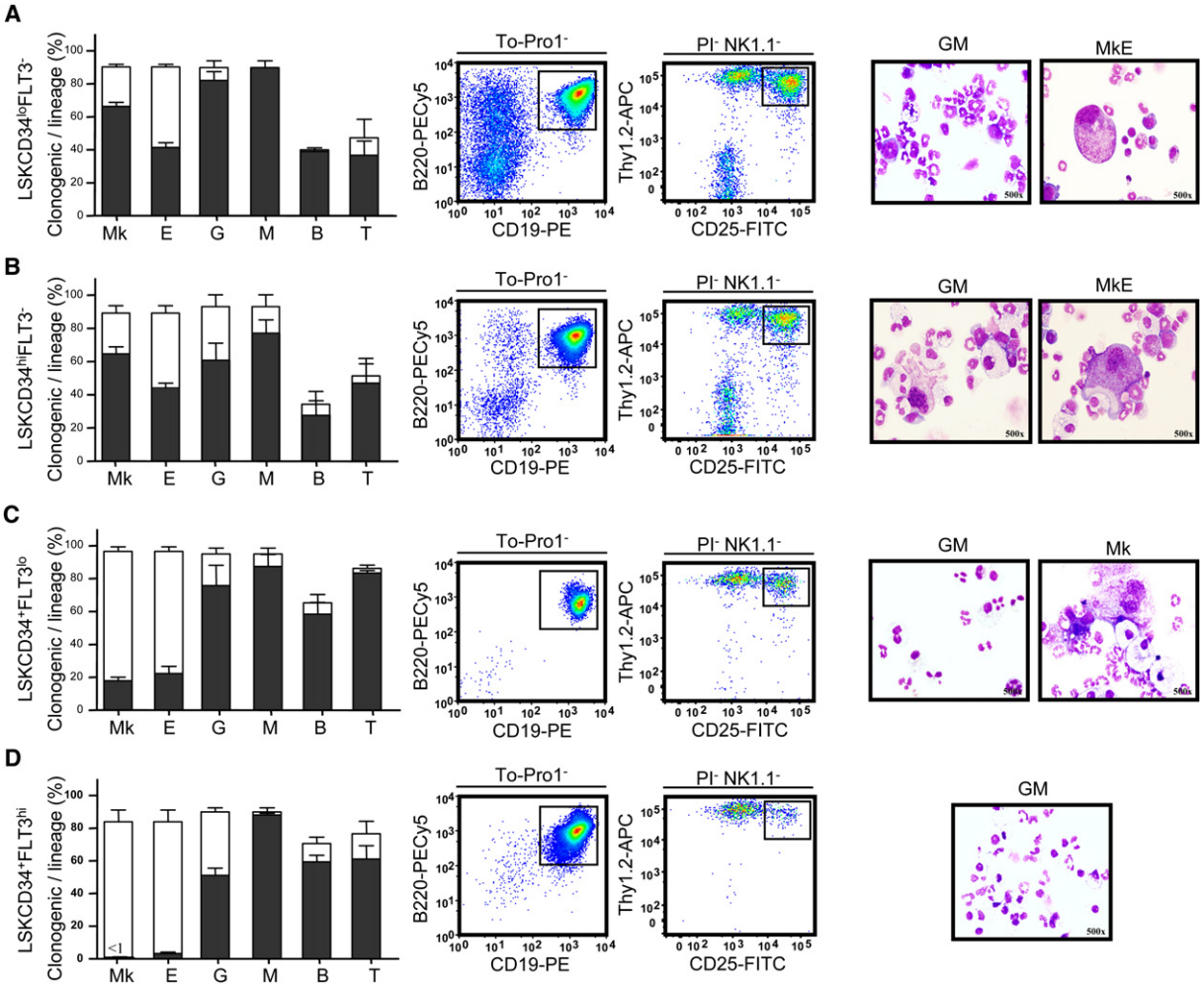


**Figure 2. LSKCD34$^+$ FL Cells Lose MkE Potential upon Upregulation of FLT3 Expression**

Single FL LSKCD34$^{lo}$FLT3$^-$ (A), LSKCD34$^{hi}$FLT3$^-$ (B), LSKCD34$^+$FLT3$^{lo}$ (C), and LSKCD34$^+$FLT3$^{hi}$ (D) cells were evaluated for their potential to develop Mk, E, G, M, B, and T cells in vitro. The potentials were investigated at multiple time points to best establish the optimal timing for each lineage and cell population (Experimental Procedures). Based on this, Mk and E potentials were evaluated at day 8 for LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$ cells and at day 6 for LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$ cells, whereas GM potential was evaluated at day 12 for LSKCD34$^{lo}$FLT3$^-$ cells, day 10 for LSKCD34$^{hi}$FLT3$^-$ cells, and day 6 for LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$ cells. Open bars show frequencies of clones formed and closed bars show frequencies of cells with indicated lineage potential. Mean ± SEM percent values from 3–6 experiments. Also shown are representative FACS profiles defining B cell (B220$^+$CD19$^+$) and T cell (NK1.1$^-$Thy1.2$^{hi}$CD25$^{hi}$) potentials, as well as representative cell morphology pictures from the GM and Mk culture conditions.

LSKCD34$^+$FLT3$^{hi}$ cells generated hematopoietic clones. Of these clones, as many as 48% (38% of plated cells; Figure 3B) contained all three lineages (BTM; Figures 3B and 3C), unequivocally demonstrating that FL LSKCD34$^+$FLT3$^{hi}$ cells largely represent LMPPs with combined B, T, and GM potentials. Interestingly, an additional 12% of FL LSKCD34$^+$FLT3$^{hi}$ cells produced B and GM cells (BM) but no (by FACS) detectable T lymphocytes, another 1% produced T and GM cells (TM) but no detectable B cells, and 8% only B and T cells (BT) in the absence of myeloid cells (Figure 3B).

The finding of FL LSKCD34$^+$FLT3$^{hi}$ cells generating BM, TM, and BT restricted clones could be compatible with the existence of BM, TM, and BT restricted progenitors, as previously proposed (Katsura, 2002). However, we speculated that it was equally possible that these BM, TM, and BT clones might at least in part rather reflect the lineage skewing of a fraction of FL LSKCD34$^+$FLT3$^{hi}$ cells with BTM potential and/or the inability to detect low levels of lineage-committed cells by FACS. We therefore next performed PCR analysis to potentially detect expression of B, T, and myeloid cell-specific genes to establish whether seemingly BM, TM, and BT cell restricted clones derived from FL LSKCD34$^+$FLT3$^{hi}$ cells might in fact contain low levels of the third lineage as well. As expected, control GM colonies were positive for the myeloid gene myeloperoxidase (*Mpo*) but negative for expression of the B cell-specific immunoglobulin lambda-like polypeptide 1 (*Igll1*)

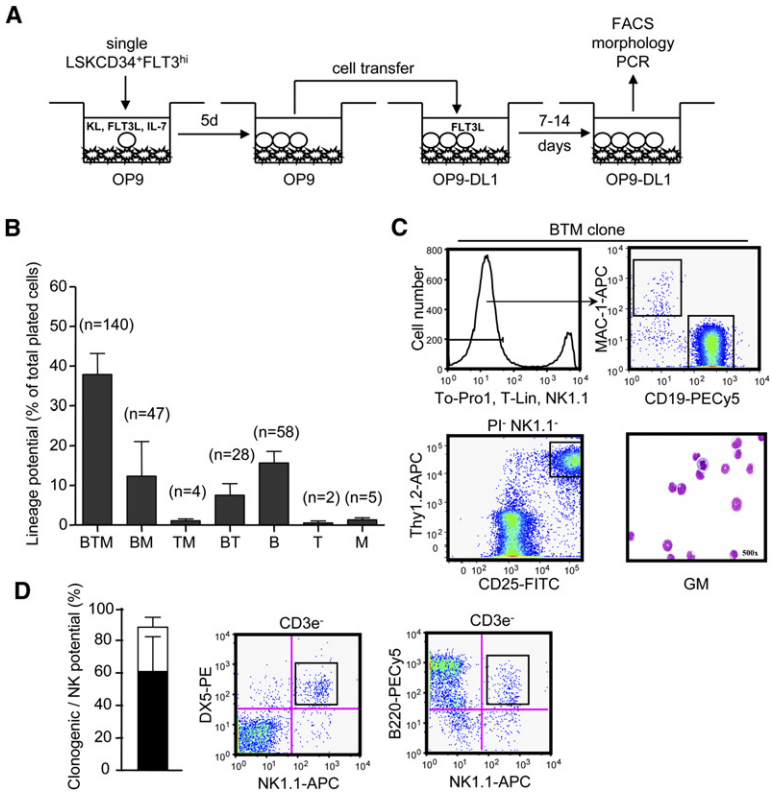


**Figure 3. Single LSKCD34$^+$FLT3$^{hi}$ FL Cells Have Combined B, T, and GM Potentials**

(A) Experimental design (for details see Experimental Procedures).

(B) Frequencies of single LSKCD34$^+$FLT3$^{hi}$ cells possessing different lineage potentials based on FACS and morphology. Mean ± SEM percent values from four experiments. Numbers shown in brackets denote the total number of clones with the specified lineage combinations.

(C) A single representative BTM clone analyzed by FACS and morphology having combined B, T, and GM potential. All lineages were confirmed with lineage-specific gene-expression analysis (Table S1, clone 9). FACS profiles show representative myeloid cells (MAC-1$^+$ and negative for CD19, NK1.1, CD3, CD4, CD8, CD25, and Thy1.2), B cells (CD19$^+$), and T cells (NK1.1$^-$Thy1.2$^{hi}$CD25$^{hi}$).

(D) NK cell potential (NK1.1$^+$DX5$^+$CD3$^-$ and/or NK1.1$^+$B220$^+$CD3$^-$) of single FL LSKCD34$^+$FLT3$^{hi}$ cells grown for 14 days on OP9 in the presence of KL, FLT3L, and IL-7. Open bar shows cloning frequency and closed bar frequency of cells with NK potential. Representative FACS profiles are shown. Mean ± SEM percent values from four experiments.

and paired box gene 5 *(Pax5)* genes and T cell-specific CD3 antigen, epsilon polypeptide *(Cd3e)*, and pre-T cell antigen receptor α *(Ptcra)* genes (Table S1). Importantly, BTM clones investigated confirmed the expression of genes for all three lineages (Table S1). Notably, of investigated BM clones, a significant fraction expressed *Cd3e* and/or *Ptcra* in addition to *Igll1*, *Pax5*, and *Mpo,* and similarly a fraction of TM as well as BT clones expressed genes for all three lineages, strongly suggesting that the frequency of cells with combined BTM potential within the FL LSKCD34$^+$FLT3$^{hi}$ population might be even higher than the 38% verified by FACS. These data demonstrate, at the single-cell level, that a substantial fraction of LSKCD34$^+$FLT3$^{hi}$ cells in FL represent LMPPs with a combined B, T, and GM but little or no MkE potential. Notably, we also found that 61% of single FL LSKCD34$^+$FLT3$^{hi}$ cells generated NK1.1$^+$DX5$^+$CD3$^-$ and/or NK1.1$^+$B220$^+$CD3$^-$ cells (Figure 3D), demonstrating their high NK cell potential.

## Hierarchical Multilineage Transcriptional Priming in Adult LSK Subsets

Because the existence of functionally defined LMPPs and their implications for the roadmap of HSC lineage commitment remains contentious (Forsberg et al., 2006; Laiosa et al., 2006), we next sought to obtain evidence for multilineage transcriptional priming of adult and fetal LMPPs, distinct from that of HSCs (Hu et al., 1997).

When compared to LSKCD34$^-$FLT3$^-$ LT-HSCs and LSKCD34$^+$FLT3$^-$ ST-HSCs, LSKCD34$^+$FLT3$^{hi}$ LMPPs in BM are by far most efficient at rapidly reconstituting the B and T cell lineages (Yang et al., 2005). In agreement with this, we previously identified by single-cell PCR, a small fraction (6%) of LMPPs that coexpressed the lymphoid interleukin 7 receptor (*Il7r*) and the myeloid colony-stimulating factor 3 receptor *(granulocyte) (Csf3r)* genes (Adolfsson et al., 2005). However, the extent of lymphoid priming and potential coexpression of distinct GM, MkE, B cell, and T cell programs within the LMPPs were not established in these and other studies of LMPPs (Lai et al., 2006; Yoshida et al., 2006), and the multilineage priming of BM LSKCD34$^-$FLT3$^-$ and LSKCD34$^+$FLT3$^-$ cells was not investigated. To better identify and understand the molecular events preceding the first lineage commitment steps of adult HSCs and LMPPs, we first purified LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^{hi}$ cells from adult BM and subjected these to Affymetrix-based global gene expression profiling. Analysis showed distinct differences in gene-expression patterns between the three LSK subpopulations (Figure S5). To avoid bias in genes classified as lineage associated, we defined MkE, GM, and lymphoid-associated genes based on global gene profiling of Mk, E, or GM differentiated FDCP-mix cells (Bruno et al., 2004) and B cell progenitor cell lines (Tsapogas et al., 2003) (Experimental Procedures, for complete lists see Figure S6). MkE-associated genes were more highly expressed in LSKCD34$^-$FLT3$^-$ than in LSKCD34$^+$FLT3$^-$ cells, and further clearly downregulated in LSKCD34$^+$FLT3$^{hi}$ LMPPs (Figure 4, Figure S6). In contrast, lymphoid-associated genes were consistently upregulated in LSKCD34$^+$FLT3$^{hi}$

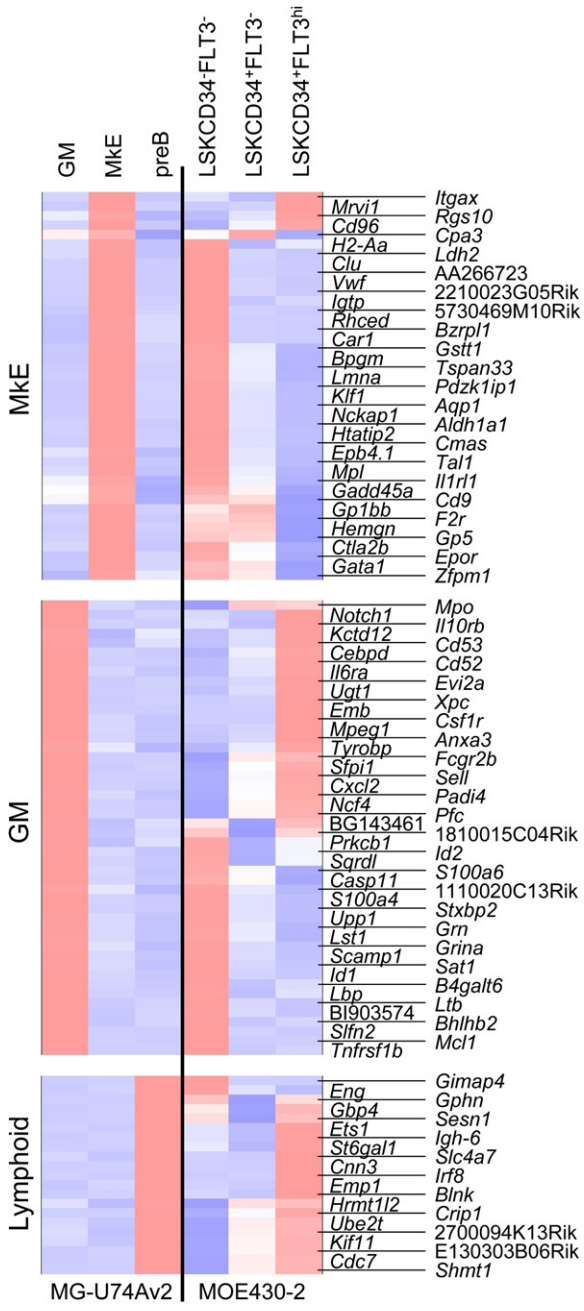


**Figure 4. Hierarchical Clustering of MkE, GM, and Lymphoid-Associated Genes in Adult BM LMPPs and HSCs**

Clusters show MkE, GM, and lymphoid-associated genes having an expression of ≥50 units in at least one LSK subpopulation and being regulated ≥2-fold between any two LSK subpopulations. Note that expression levels are not directly comparable between MG-U74Av2 and MOE430 2.0 chip data. Red indicates high, blue low, and white intermediate expression levels. (For complete lists of lineage-associated genes and cluster with indicated expression values, see Figure S6.)

LMPPs versus LSKCD34$^+$FLT3$^-$ and LSKCD34$^-$FLT3$^-$ cells (Figure 4; Figure S6), whereas GM-associated genes were apparently expressed in all three LSK populations. A similar picture of differences in lineage-associated genes was obtained when doing the comparison directly based on the differentially regulated genes observed between the LSK subpopulations (Figure S7).

We next used quantitative RT-PCR (Q-RT-PCR) to investigate the expression patterns for a number of specific transcription factors, cytokine receptors, and other regulators considered to play key roles in regulating the MkE, GM, and lymphoid lineages. The MkE-associated genes GATA binding protein 1 (*Gata1*), erythropoietin receptor (*Epor*), and myeloproliferative leukaemia virus oncogene (*Mpl*; receptor for thrombopoietin) were all expressed at the highest levels in BM LSKCD34$^-$FLT3$^-$ cells, reduced in LSKCD34$^+$FLT3$^-$ cells, and clearly downregulated or turned off in LSKCD34$^+$FLT3$^+$ LMPPs (Figure 5A). Similar results were obtained for *growth factor independent 1B* (*Gfi1b*) and GATA binding protein 2 (*Gata2*) genes, both displaying reduced expression in BM LMPPs as compared to LSKCD34$^+$FLT3$^-$ cells (Figure 5A). In contrast, the genes for the important GM regulators *Csf3r* and SFFV proviral integration 1 (*Sfpi1*) as well as CCAAT/enhancer binding protein α (*Cebpa*) and colony-stimulating factor 1 receptor (*Csf1r*) were expressed at comparable levels in the different BM LSK subpopulations (Figure 5B).

We also investigated the status of B and T cell-associated genes, as well as genes known to be expressed in both early committed B and T cell progenitors. Notably, in agreement with the global analysis, B cell (*Pax5* and *Igll1*) and T cell (Ptcra and *Cd3e*) lineage-specific genes were not detectable in any of the BM LSK subpopulations (Figure 5C), including the LMPP, distinguishing them from CLPs that coexpress B and T cell genes (Kondo et al., 1997). In contrast, all four investigated lymphoid genes (recombination activating gene 1 and 2 [*Rag1, Rag2*], *deoxynucleotidyltransferase, terminal* [*Dntt*], and sterile IgH transcript) thought to be expressed prior to B and T cell specification were upregulated in LSKCD34$^+$FLT3$^{hi}$ LMPPs (Figure 5D). Thus, global gene profiling demonstrates a hierarchical pattern of transcriptional lineage priming with downregulation of MkE programs and upregulation of early lymphoid programs from enriched adult HSC to LMPP populations.

## Combined Lymphoid and GM but Not MkE Priming in Single Adult LMPPs

Although the global and Q-PCR analyses demonstrated a distinct hierarchical pattern of MkE, GM, and common lymphoid transcriptional priming within the BM LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^{hi}$ populations, these analyses could not establish the frequency of cells expressing each lineage program nor the extent to which distinct lineage programs are co-expressed within the same or reside in distinct cells within each of the three LSK populations. This represents a considerable limitation of such population-based molecular analysis, as shown by the fact that any phenotypically purified stem or progenitor populations also reveal heterogeneous biological potentials. We therefore performed multiplex single-cell RT-PCR analysis (Hu et al., 1997) of each of the three BM populations by using primers for

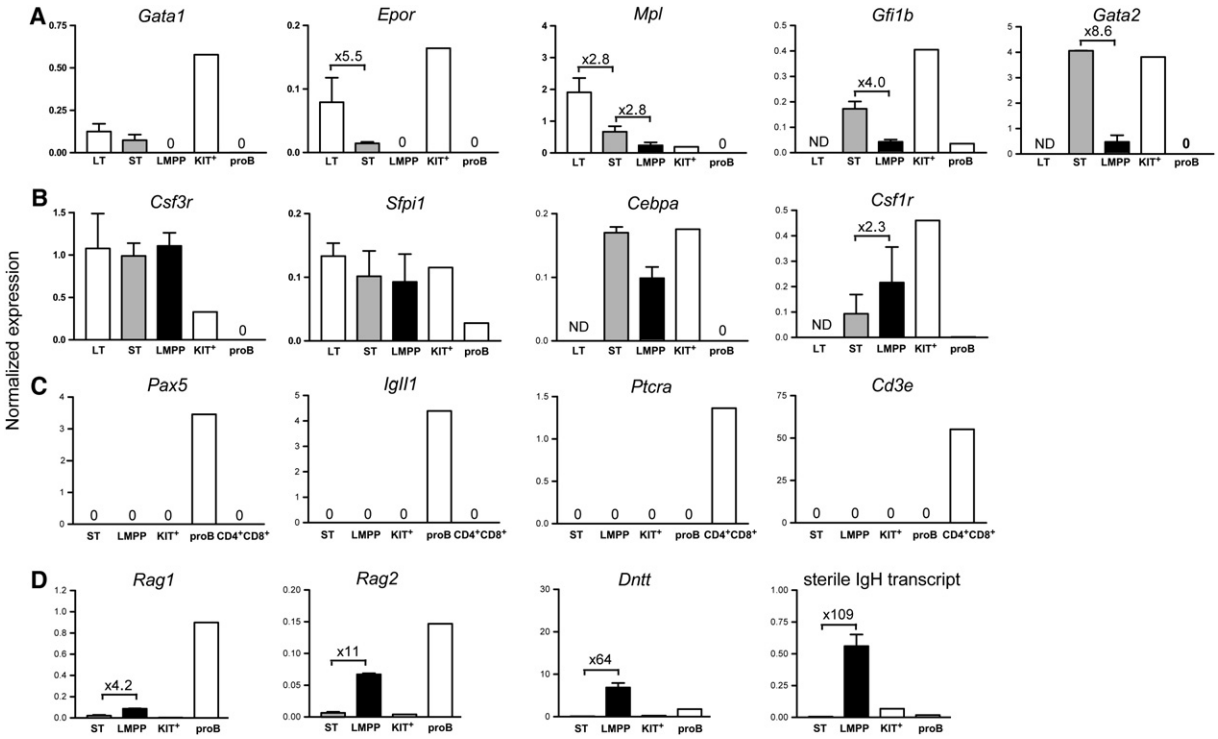


**Figure 5. Expression Levels of MkE, GM, and Lymphoid Genes in Adult BM LMPPs and HSCs**

Quantitative PCR analysis of expression of MkE (A), GM (B), B and T cell (C), and common lymphoid (D) genes in FACS-purified BM LSKCD34$^-$FLT3$^-$ LT-HSCs (LT), LSKCD34$^+$FLT3$^-$ ST-HSCs (ST), and LSKCD34$^+$FLT3$^{hi}$ LMPPs (LMPP). BM Lin$^-$ KIT$^+$ cells (containing progenitor cells of different lineages), BM CD43$^+$AA4.1$^+$CD19$^+$ (pro-B) cells, and CD4$^+$CD8$^+$ thymocytes were used as controls. All data were normalized to the expression of hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*). Mean ± SEM percent values from at least two different sorts. Q-PCR measurements were done in triplicates. 0 = no detectable expression after 45 cycles of PCR. Mean differences between LSK subsets higher than 2-fold are indicated. ND = not done.

2–3 genes characteristic of each lineage. The genes selected (MkE: *Gata1, Epor*, and Von Willebrand factor homolog [*Vwf*]; GM: *Csf3r* and *Mpo*; and lymphoid: *Il7r,* sterile IgH transcript, and *Rag1*) were chosen on the basis of their known specific roles and/or expression patterns in each of these lineages at the postcommitment level. Importantly, we first confirmed the restricted expression pattern of each of these genes by analyzing their expression in different purified committed myeloid and lymphoid progenitor cells (Table S2). Experiments also showed that these primers detect expression of the gene of interest at a very high frequency in committed cells expected to be positive (Table S2).

The single-cell analysis confirmed the hierarchical pattern of lineage priming observed by global gene profiling and Q-PCR by demonstrating the highest frequency of cells expressing MkE-associated genes in BM LSKCD34$^-$FLT3$^-$ cells (37%), less in LSKCD34$^+$FLT3$^-$ cells (23%), and virtual absence of such cells in LSKCD34$^+$FLT3$^{hi}$ LMPPs (<1%; Figure 6A). In contrast, investigated GM genes were expressed in as much as 81% and 93% of LSKCD34$^+$FLT3$^-$ and LSKCD34$^+$FLT3$^{hi}$ cells, respectively, as compared to only 33% in LSKCD34$^-$FLT3$^-$ cells (Figure 6A). Finally, although virtually no LSKCD34$^-$FLT3$^-$ or LSKCD34$^+$FLT3$^-$ cells expressed any of the lymphoid genes investigated, as many as 31% of LSKCD34$^+$FLT3$^{hi}$ LMPPs did (Figure 6A).

The most compelling evidence for a hierarchical expression of MkE, GM, and lymphoid lineage genetic programs was observed when investigating their coexpression patterns (Figure 6A). MkE and GM programs were coexpressed in LSKCD34$^-$FLT3$^-$ and LSKCD34$^+$FLT3$^-$ cells. Similarly, combined GM and lymphoid priming was observed in LSKCD34$^+$FLT3$^{hi}$ cells, with virtually all lymphoid-primed cells also coexpressing GM genes. In striking contrast, no BM LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, or LSKCD34$^+$FLT3$^{hi}$ LMPPs were found to have combined expression of the investigated MkE and lymphoid genes.

Finally, we also investigated the coexpression of the different lineage programs with *Mpl,* shown to play a dual role in hematopoiesis, being critical for maintenance and development of not only Mk progenitors but also HSCs (Buza-Vidas et al., 2006; Kimura et al., 1998). As expected (Buza-Vidas et al., 2006), the highest frequency of *Mpl* expression was observed in BM LSKCD34$^-$FLT3$^-$ cells (59%) followed by LSKCD34$^+$FLT3$^-$ cells (42%), but also a smaller fraction (17%) of LSKCD34$^+$FLT3$^{hi}$ LMPPs expressed *Mpl* (Figure 6B). Notably, virtually all of the *Mpl*-positive LSKCD34$^+$FLT3$^{hi}$ cells were GM but not

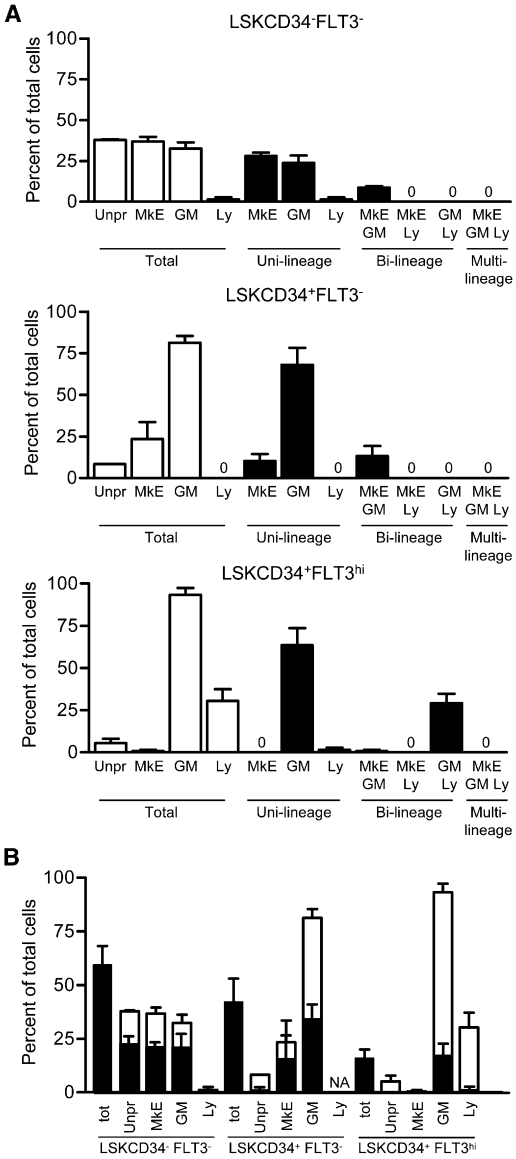


**Figure 6. Hierarchical Coexpression Patterns of MkE, GM, and Lymphoid Lineage Programs in Adult LSK Subsets**

(A) Distribution of lineage programs.

(B) Coexpression of *Mpl* with different lineage programs. Open bars show frequencies of cells within each LSK population having indicated lineage priming, and black bars frequencies of cells coexpressing specific lineage programs and *Mpl*. Expression of *Kit* served as an internal positive control for the RT-PCR, thus only cells being *Kit* mRNA positive were further analyzed (typically ~90% of investigated BM LSK cells). Cells were scored as expressing MkE, GM, and/or lymphoid programs based on the expression of one or more lineage-associated genes: MkE, *Gata1, Vwf,* and *Epor*; GM, *Csf3r* and *Mpo*; lymphoid, *Rag1,* sterile IgH transcript, and *Il7r*. Cells were, based on expressed programs, classified as: unprimed (unpr), having no lineage program; uni-lineage, having one program; bi-lineage, having any two programs; or multilineage, having all programs. Mean ± SEM percent values from two independent experiments with 88 single cells analyzed in each experiment. NA, nonapplicable (as no cells of indicated phenotype expressed the specified lineage program).

lymphoid primed, again demonstrating a lack of coexpression of MkE and lymphoid lineage programs. β-globin (*Hbb* family), an MkE-associated gene family, was also expressed at low frequencies in LSKCD34$^+$FLT3$^{hi}$ cells, but as for *Mpl* almost exclusively together with the GM and not lymphoid genes (R.M., A.H., and S.E.W.J., unpublished data). Thus, adult BM LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^{hi}$ LMPPs represent hierarchically related subpopulations within the LSK HSC compartment with distinct patterns of multilineage transcriptional priming, most notable being the upregulation of common lymphoid programs in LMPPs at a stage where MkE priming is lost but the GM program sustained, matching the upregulation of lymphoid and loss of MkE lineage potentials in adult LMPPs (Adolfsson et al., 2005).

## Hierarchical Transcriptional Priming Is Established during Fetal Development

The distinct pattern of lineage potentials of FL LSKCD34$^+$FLT3$^{hi}$ cells suggested that LMPPs are defined already in fetal development. If so, we would also predict a similar and distinct pattern of lineage transcriptional priming in fetal LMPPs as observed in their adult counterparts. Thus, we next investigated FL LSKCD34$^+$FLT3$^{hi}$ when compared to LSKCD34$^{lo}$FLT3$^-$, LSKCD34$^{hi}$FLT3$^-$, and LSKCD34$^+$FLT3$^{lo}$ cells, because adult LMPPs coexpress GM and lymphoid but not MkE genes. One notable difference from the adult BM (Figure 6) was that a much higher fraction of each FL LSK population was multilineage transcriptional primed and virtually no unprimed cells were observed (Figure 7). However, as for the distinct hierarchical organization of MkE, GM, and lymphoid programs observed in adult BM, an almost identical pattern was observed in the FL. Specifically, MkE priming was most prominent in LSKCD34$^{lo}$FLT3$^-$ cells (70%) and gradually downregulated to become very rare (9%) in LSKCD34$^+$FLT3$^{hi}$ cells. GM priming was high (83%–97%) in all populations, whereas lymphoid priming was exceptionally rare (<1%) in LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$ cells, observed in 14% of LSKCD34$^+$FLT3$^{lo}$ cells and in as much as 63% of single LSKCD34$^+$FLT3$^{hi}$ LMPPs. MkE and GM programs were typically coexpressed in LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$ cells, and almost all lymphoid-primed LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$ LMPPs were also GM primed (Figure 7). As in adult BM, not a single FL LSK cell in any of the three populations coexpressed the investigated MkE and lymphoid genes without also being GM primed. However, at variance with adult BM, very rare FL LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$ cells did coexpress all three (MkE, GM, and lymphoid) programs (3% and 6%, respectively; Figure 7). It is noteworthy that all of these cells proved to express only one of the three investigated MkE genes (see Figure S8 for breakdown of coexpression patterns). Thus, multilineage gene-expression analysis at the global and single-cell level demonstrate in adult as well as fetal LSKCD34$^+$FLT3$^{hi}$ LMPPs a multilineage transcriptional priming pattern

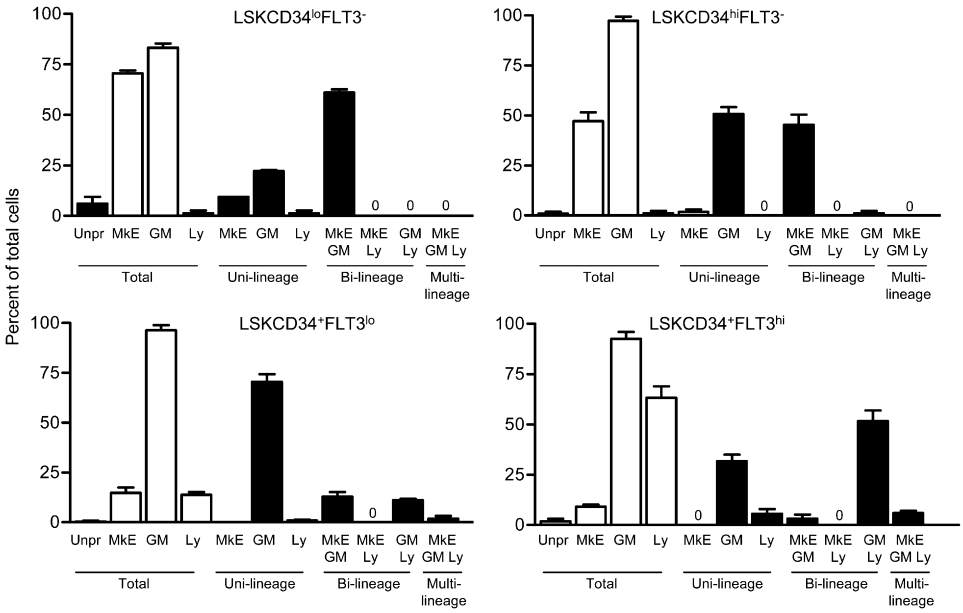


**Figure 7. Hierarchical Organization of MkE, GM, and Lymphoid Programs within Distinct Fetal LSK Subpopulations**

Coexpression patterns of lineage programs in single cells from fetal LSK subpopulations. Cells were scored as expressing MkE, GM, and/or lymphoid programs based on the expression of one or more lineage-associated genes: MkE, *Gata1, Vwf,* and *Epor*; GM, *Csf3r,* and *Mpo*; lymphoid, *Rag1,* sterile IgH transcript, and *Il7r*. Mean ± SEM percent values from two to three independent experiments, with 88 cells of each population investigated in each experiment.

unambiguously matching their loss of MkE and upregulation of lymphoid potentials.

## DISCUSSION

Although the existence of distinct CMPs and CLPs is compatible with the first lineage commitment step of pluripotent adult HSCs resulting in a strict separation of common myeloid and common lymphoid commitment pathways, it does not preclude that lineage commitment might also occur through alternative cellular intermediates. A model for adult hematopoiesis in which CMPs and CLPs are obligatory intermediates for lineage commitment (Reya et al., 2001) would predict that each of the LT-HSCs, ST-HSCs, and MPPs would differ only in their self-renewal capacity but sustain the same repertoire of lineage potentials.

In the present study we established the multilineage molecular priming of recently identified distinct and hierarchical related adult BM LSKCD34$^{-}$FLT3$^{-}$, LSKCD34$^{+}$FLT3$^{-}$, and LSKCD34$^{+}$FLT3$^{hi}$ cells and provided evidence for a hierarchical organization of transcriptional lineage priming within these multipotent HSC and progenitor compartments. Most notably, and in agreement with the functional data for lineage potentials (Adolfsson et al., 2005), LSKCD34$^{+}$FLT3$^{hi}$ LMPPs when compared to HSCs downregulate the MkE and upregulate the lymphoid gene expression while sustaining GM priming. The typical lymphoid-primed genes in LMPPs are those expressed prior to B and T cell commitment. Further, multiplex single-cell PCR demonstrated that virtually every adult lymphoid-primed LMPP coexpresses GM genes, in contrast to committed T and B cell progenitors as well as CLPs (Kondo et al., 1997; Miyamoto et al., 2002). Whereas previous studies had demonstrated that enriched populations of MPPs, unlike HSCs, express a limited repertoire of lymphoid genes (Akashi et al., 2003), the present study at the single-cell level unequivocally establishes that this lymphoid priming occurs in a population of LMPPs that coexpress GM but no MkE genes, and which at the functional level sustains combined GM and lymphoid but little or no MkE lineage potential (Adolfsson et al., 2005). Most noteworthy, combined lymphoid and MkE transcriptional priming was never observed in single adult LMPPs (or LT-HSCs and ST-HSCs).

Previous descriptions of lympho-myeloid lineage-restricted MPPs in FL, with a combined B, monocyte (Cumano et al., 1992), and in some cases also T cell (Katsura, 2002; Lacaud et al., 1998) potential, had implicated potentially distinct pathways for HSC lineage commitment in FL and adult BM. However, our current and comparative studies of BM and FL demonstrate that the recently described GM-lymphoid-primed LSKCD34$^{+}$FLT3$^{hi}$ LMPP in adult BM (Adolfsson et al., 2005; Yang et al., 2005) is defined already during fetal development and that the distinct hierarchy of transcriptional lineage priming in the adult LSK hierarchy is established already in the FL. In FL LSKCD34$^{+}$FLT3$^{hi}$ LMPPs, a very low frequency of cells with coexpression of MkE and lymphoid genes was observed, but without exception these were, as in adult BM, also GM primed and expressed only one of the investigated MkE genes. This could potentially reveal a rare

multipotent FL LSKCD34$^+$FLT3$^{hi}$ progenitor in active transition from MkE to lymphoid priming, or a LMPP recently generated from HSCs with retention of some residual mRNA for single MkE genes. This finding is also compatible with a low frequency of BM LSKCD34$^+$FLT3$^{hi}$ cells (<2%–3%) having residual MkE potential, as previously reported (Adolfsson et al., 2005; Forsberg et al., 2006). Regardless, through extensive studies in BM and FL, we found compelling evidence for the existence of frequent LSKCD34$^+$FLT3$^{hi}$ LMPPs with combined priming of GM and lymphoid but not MkE gene-expression programs, unambiguously linking their combined GM and lymphoid lineage potentials with GM-lymphoid-restricted gene expression.

With the OP9 and OP9-DL1 stromal cell lines, as much as 51% of single FL LSKCD34$^+$FLT3$^{hi}$ cells generated both GM and lymphoid progeny based on stringently validated FACS and morphology criteria. The by far most common GM-lymphoid clone produced from single LSKCD34$^+$FLT3$^{hi}$ cells produced the GM and B as well as T cell lineages, establishing the existence of FL LSKCD34$^+$FLT3$^{hi}$ LMPPs. Based on FACS analysis, we also observed BM- and TM- as well as BT-restricted clones. However, PCR analysis for lineage-specific genes suggested that many of these clones might also produce the third lineage not detected by FACS, so the emergence of these seemingly lineage-restricted clones could alternatively reflect, at least in part, the inability of the utilized assays to optimally and simultaneously promote and uncover all the lineage potentials of every LSKCD34$^+$FLT3$^{hi}$ cell investigated. Whereas we, in our studies of adult BM, investigated only LSK cells with the highest levels of FLT3 expression (LSKCD34$^+$FLT3$^{hi}$) (Adolfsson et al., 2005), we here also investigated the FL LSKCD34$^+$FLT3$^{lo}$ population for its pattern of lineage priming and potentials, and we made several important observations. Based on their repopulating ability and their lineage potentials, LSKCD34$^+$FLT3$^{lo}$ cells represent an intermediate population between LSKCD34$^{hi}$FLT3$^-$ and LSKCD34$^+$FLT3$^{hi}$ cells, a conclusion also supported by having intermediate levels of MkE and lymphoid priming and lineage potentials, but importantly MkE and lymphoid programs were also virtually mutually exclusive within single LSKCD34$^+$FLT3$^{lo}$ cells and never coexpressed in the absence of GM priming.

The seminal discovery of HSC-enriched populations being uniquely multilineage primed suggested that HSCs express low levels of important genes for multiple lineages prior to lineage commitment (Hu et al., 1997), but its biological significance has remained unclear. Through detailed functional and gene-expression analysis at the single-cell level of distinct and hierarchical related HSC and MPP populations in FL and adult BM, the present studies represent a step forward in understanding the implications of multilineage transcriptional priming, first by uncovering a further complexity as to the patterns of multilineage priming observed in phenotypically and functionally distinct HSC and MPP subsets, and second by demonstrating how the different lineage programs are in fact hierarchically organized within each of these compartments, with the GM program perhaps being the default lineage program throughout the multipotent LSK hierarchy. Taken together with the molecular and functional data presented here and in other studies (Adolfsson et al., 2005; Lai et al., 2006; Yoshida et al., 2006), our findings could be compatible with MkE-GM and subsequent GM-lymphoid (LMPPs) progenitors being generated upon commitment of pluripotent HSCs, although this remains to be demonstrated.

Notably, the multilineage priming was much more extensive in fetal than in adult HSC populations, pertaining to all investigated LSK populations, and to MkE, GM, and lymphoid genes, although importantly without changing the hierarchical pattern observed between different lineage programs and distinct populations. Although recent studies that used in vivo lineage tracing convincingly demonstrated that a small fraction of BM LT-HSCs express low levels of the myeloid gene lysozyme (*Lyzs*) (Ye et al., 2003), it has not been possible to exclude that adult LT-HSCs are predominantly unprimed, because of the high frequency of phenotypically defined adult HSCs reported to be unprimed (Akashi et al., 2003; Hu et al., 1997), confirmed for adult HSCs in our studies, combined with the inability to purify HSCs to homogeneity. However, our FL studies virtually excludes this possibility: we here find that virtually every FL LSKCD34$^{lo}$FLT3$^-$ cell is transcriptionally lineage primed, predominantly having a combined MkE and GM priming. Although this could partially reflect that adult HSCs are more quiescent, it is unlikely to provide the sole explanation, because the ontogeny-related differences in lineage priming are also observed in the more actively cycling LSKCD34$^{hi}$FLT3$^-$ and LSKCD34$^+$FLT3$^{hi}$ cells.

In conclusion, based on the hierarchical pattern of transcriptional lineage priming and the identification of GM-lymphoid restricted fetal and adult LMPPs, we propose an alternative model for HSC lineage commitment, in which no strict restriction or branching points might exist in the HSC hierarchy. Rather, a hierarchically ordered transcriptional activation and downregulation of distinct lineage programs is most compatible with the probability for undergoing different lineage commitment fates, changing gradually when progressing from LSKCD34$^-$FLT3$^-$ LT-HSCs to LSKCD34$^+$FLT3$^{hi}$ LMPPs, giving rise to preferentially MkE-GM and lymphoid-GM restricted progenitors, respectively. This model also predicts that although any HSC commitment fate might be possible, the probability of certain commitment fates, in particular MkE-lymphoid-restricted progenitors, should be exceptionally low, regardless of the stage in the HSC hierarchy. Importantly, although this model implicates that the previously identified CMPs and CLPs might not represent obligatory or even main intermediates in hematopoietic lineage commitment, it is fully compatible with the existence of CMPs and CLPs. Although this proposed model will need to be substantiated by direct experimental evidence, it is supported by the kinetics of different blood cell lineages emerging during evolution, ontogeny, and

post-transplantation (Cumano and Godin, 2001; Hansen and Zapata, 1998; Yang et al., 2005).

## EXPERIMENTAL PROCEDURES

### FACS Purification of LSK HSC Subpopulations from BM and FL

BM cells were harvested from 11- to 14-week-old C57Bl/6 mice, and LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^{hi}$ populations were FACS purified as previously described (Adolfsson et al., 2005; Yang et al., 2005), resulting in 98% or higher purity of each population (Figure S1). Lineage depleted (Lin$^-$) or KIT-enriched E14.5 FL cells (Supplemental Experimental Procedures) were stained against SCA-1, KIT, CD34, and FLT3 as described (Adolfsson et al., 2005; Yang et al., 2005), with the exception that biotinylated SCA-1 was visualized with Streptavidin-PE-TexasRed or Streptavidin-PE-Cy7. Cells were sorted for indicated phenotypes on a BD FACSDiVa or BD FACSAria (BD Biosciences, San Jose, CA).

### Affymetrix Gene Expression and Data Analysis

RNA was extracted as previously described for RT-Q-PCR (Adolfsson et al., 2005), labeled, amplified, and hybridized to MOE430 2.0 Affymetrix gene expression arrays according to Affymetrix standard protocols (Supplemental Experimental Procedures). Probe level expression values were calculated with RMA (Irizarry et al., 2003). Based on previous gene-expression profiles of pre-B cell lines (Tsapogas et al., 2003) and multipotent FDCP-mix cells (7 days after induction of differentiation toward either the GM, Mk, or E lineage) (Bruno et al., 2004), lineage-associated genes were defined (among genes having an expression of $\geq$100 in one group of arrays and with a lower 90% confidence bound of fold change) as MkE: (Mk$^{expr}$/GM$^{expr}$ $\geq$ 2 and Mk$^{expr}$/pre-B$^{expr}$ $\geq$ 2) or (E$^{expr}$/GM$^{expr}$ $\geq$ 2 and E$^{expr}$/pre-B$^{expr}$ $\geq$ 2); GM: (GM$^{expr}$/Mk$^{expr}$ $\geq$ 2 and GM$^{expr}$/pre-B$^{expr}$ $\geq$ 2 and GM$^{expr}$/E$^{expr}$ $\geq$ 2); Lymphoid: (pre-B$^{expr}$/GM$^{expr}$ $\geq$ 2 and pre-B$^{expr}$/Mk$^{expr}$ $\geq$ 2 and pre-B$^{expr}$/E$^{expr}$ $\geq$ 2) (where Mk$^{exp}$, E$^{expr}$, GM$^{expr}$, and pre-B$^{expr}$ is the mean expression value in arrays representing that lineage). For complete sets of genes classified as lineage associated, see Figure S6. Matching of MOE430 2.0 to MG-U74Av2 probe sets (with best match array comparison spreadsheets provided by Affymetrix) and further analysis was done with dChip (http://biosun1.harvard.edu/complab/dchip/). Array data are accessible through the gene expression omnibus (GEO; GSE7302).

### Quantitative RT-PCR

RNA extraction and RT-Q-PCR of adult BM LSKCD34$^-$FLT3$^-$, LSKCD34$^+$FLT3$^-$, and LSKCD34$^+$FLT3$^{hi}$ cells was performed as previously described (Adolfsson et al., 2005). For TaqMan probes used, see Supplemental Experimental Procedures.

### Gene-Expression Analysis of Single Cells by Multiplex RT-PCR

Multiplex single-cell RT-PCR analysis was performed as previously described (Adolfsson et al., 2005; Hu et al., 1997). See Supplemental Experimental Procedures for details.

### In Vivo Multilineage Reconstitution Assay for FL LSK Subpopulations

Mouse experiments were approved by the ethical committee at Lund University. Competitive reconstitution assays with congenic CD45.1/CD45.2 mice were performed as previously described (Yang et al., 2005). Peripheral blood (PB) reconstitution levels were analyzed by staining for CD45.1, CD45.2, and B cell (B220), T cell (CD4 and CD8), and myeloid (MAC-1) cell-surface antigens (see Supplemental Experimental Procedures for antibody information). Secondary transplantations were performed as previously described (Bryder and Jacobsen, 2000). For each lineage, >0.02% test cell contribution (and >10 positive test cell-derived events within correct scatter profile) toward total PB cells was required to define mice as reconstituted. For total reconstitution, mice were considered positive if one or more lineages fulfilled the above criteria. Percentage of donor (test cell)-derived reconstitution was calculated as: (% donor cells)/(% donor + % support cells).

### In Vitro Evaluation of MkE, GM, and Lymphoid Potentials of FL LSK Subpopulations

For evaluating MkE potential, a previously described method (Adolfsson et al., 2005) was slightly modified. In brief, single cells were seeded (Supplemental Experimental Procedures) in 60-well plates (Nunc Minitrays catalog number: 163118, Nunc A/S, Roskilde, Denmark) in 20 μL X-vivo 15 (BioWhittaker, Walkersville, MD), 0.5% detoxified bovine serum albumin (BSA; StemCell Technologies Inc., Vancouver, Canada) and 10% fetal calf serum (FCS, BioWhittaker, Verviers, Belgium, or Sigma-Aldrich, St. Louis, MO), supplemented with cytokines (50 ng/mL KIT ligand [KL], 50 ng/mL fms-like tyrosine kinase 3 ligand [FLT3L], 50 ng/mL thrombopoietin [THPO], 5 U/mL erythropoietin [EPO], and 20 ng/mL interleukin 3 [IL-3]; for detailed cytokine information see Supplemental Experimental Procedures). Wells were scored, with an inverted light microscope, for clonal growth and frequency of Mk and E cells after 4 (for LSKCD34$^+$FLT3$^{lo}$, LSKCD34$^+$FLT3$^{hi}$), 6, 8, 10 (all populations), and 12 (LSKCD34$^{lo}$FLT3$^-$, LSKCD34$^{hi}$FLT3$^-$) days of culture, to establish optimal time point for MkE evaluation (see Figure S3 for LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$ cells, data not shown for LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$ subpopulations). Mks were verified morphologically on May-Grünwald Giemsa (MGG)-stained cytospin preparations. E potential was evaluated by 2,7-diaminofluorene (DAF; Sigma-Aldrich) staining (Kaiho and Mizuno, 1985) (see Supplemental Experimental Procedures).

For evaluating GM potential, single cells were seeded in 60-well plates in 20 μL X-vivo 15 supplemented with 0.5% BSA and cytokines (KL, FLT3L, THPO, granulocyte-macrophage colony-stimulating factor [GM-CSF], granulocyte colony-stimulating factor [G-CSF], all 50 ng/mL and 20 ng/mL IL-3). Wells were scored, with an inverted light microscope, for clonal growth after 4 (LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$), 6, 8, 10 (all populations), and 12 (LSKCD34$^{lo}$FLT3$^-$ and LSKCD34$^{hi}$FLT3$^-$) days of culture. The optimal read-out time point for GM evaluation was established (data not shown) to be day 12 for LSKCD34$^{lo}$FLT3$^-$ cells, day 8–10 for LSKCD34$^{hi}$FLT3$^-$ cells, and day 6 for LSKCD34$^+$FLT3$^{lo}$ and LSKCD34$^+$FLT3$^{hi}$ cells. The frequency of clones containing G and M cells were scored by morphologic evaluation of MGG-stained cytospin preparations.

For evaluating B, NK, and T cell potential, single cells were seeded onto ~80% confluent monolayers of OP9 or OP9-DL1 stromal cells. OP9 stromal cocultures were supplemented with 25 ng/mL KL, 25 ng/mL FLT3L, and 20 ng/mL interleukin 7 (IL-7), whereas OP9-DL1 cocultures were supplemented with 25 ng/mL KL (only first week) and 25 ng/mL FLT3L. Half the coculture medium was replaced every week. Visible clones were picked at day 14 and 21 from OP9 and OP9-DL1 cocultures, respectively, and analyzed by FACS for B cell (B220$^+$CD19$^+$), NK cell (NK1.1$^+$DX5$^+$CD3$^-$ and/or NK1.1$^+$B220$^+$CD3$^-$), and T cell (CD4$^+$CD8$\alpha^+$ and/or NK1.1$^-$Thy1.2$^{hi}$CD25$^{hi}$) (Rumfelt et al., 2006) committed progeny, respectively. In addition, clones were required to have $\geq$20 gated events (of indicated cell-surface phenotypes) with appropriate scatter profile to be scored as positive. Small clones were cultured an additional week before being analyzed as described.

### In Vitro Combined Lineage Potentials of Single FL LSKCD34$^+$FLT3$^{hi}$ LMPPs

A switch-culture system was set up to evaluate the combined GM, B, and T potential from single LSKCD34$^+$FLT3$^{hi}$ FL cells. LSKCD34$^+$FLT3$^{hi}$ cells were seeded as single cells (Supplemental Experimental Procedures) onto a ~80% confluent OP9 monolayer in 48-well plates containing media supplemented with KL (25 ng/mL), FLT3L (25 ng/mL), and IL-7 (20 ng/mL) as described above. After 5 days of coculture, cells from each OP9-containing well were transferred to confluent OP9-DL1 monolayer wells supplemented with only FL (25 ng/mL). After an additional 7 days of coculture, individual

wells with visible clones were investigated for the presence of B cells ($CD19^+$), T cells ($CD4^+CD8\alpha^+$ and/or $NK1.1^-Thy1.2^{hi}CD25^{hi}$), and GM cells ($GR\text{-}1^+$ and/or $MAC\text{-}1^+$ and $CD19^-CD3^-CD4^-CD8^-CD25^-Thy1.2^-NK1.1^-$) by FACS. At least 20 events with the appropriate scatter profiles were required for each lineage to be considered as positive. Small clones were cultured for an additional 7 days and analyzed as described. GM potential was verified morphologically by microscopy of MGG stained cytospin preparations.

### Supplemental Data

Eight figures, two tables, and Experimental Procedures are available at http://www.immunity.com/cgi/content/full/26/4/407/DC1/.

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